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14. ABSTRACT
The overall goal of this research was to improve treatment of infected segmental bone loss associated with combat injuries. Our specific aim was to demonstrate whether human recombinant bone morphogenetic protein-2 (rhBMP-2) and antibiotic delivered locally in a composite carrier, together with administration of systemic antibiotic, could lead to new bone formation in an internally-stabilized segmental defect in a rat femur with a chronic infection from Staphylococcus aureus. It was found that rhBMP-2 maintained its osteoinductive capability despite the presence of chronic infection and colonized hardware, and this property was enhanced by local and systemic antibiotic. No significant new bone was formed unless rhBMP-2 was introduced. In one group, a composite collagen sponge/ceramic-collagen matrix carrier containing 200 µg of rhBMP-2 (sponge) and 100 mg of Cefazolin (matrix) was applied to surgically debrided defects, together with 4 weeks of systemic administration of the antibiotic Ceftriaxone. Despite the chronic infection, this treatment induced a substantial amount of newly mineralized callus that connected the ends of the defect 8 weeks after debridement such that there was no significant difference between the torsional failure strength of these treated defects and the intact contralateral femurs. All measures of healing improved over time.

15. SUBJECT TERMS
segmental defect, chronic infection, Staphylococcus aureus, bone morphogenetic protein, antibiotic, osteomyelitis, BMP-2, rat model

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Introduction

The majority of the combat casualties that occur in Operations Iraqi Freedom and Enduring Freedom are a result of high-energy blast or high-velocity projectile mechanisms, and commonly present with a significant segmental bone defect, massive soft tissue disruption and loss, and substantial contamination with bacteria. The goal of this research was to improve the treatment of infected segmental bone loss by using currently available off-the-shelf biologics and antibiotics. Specifically, the aim of this study was to determine whether recombinant human bone morphogenetic protein-2 (rhBMP-2) and antibiotic delivered locally from a composite carrier, in combination with a second antibiotic delivered systemically, could lead to new bone formation in an internally-stabilized rat femoral segmental defect with a chronic infection from *Staphylococcus aureus*. It is hypothesized that (i) chronically-infected defects treated with debridement and rhBMP-2 would form significantly more and stronger new bone than debrided defects without rhBMP-2, (ii) defects treated with debridement, rhBMP-2 and local administration of a high dose of antibiotic would form significantly more and stronger new bone than debrided defects treated with rhBMP-2 alone, and (iii) defects treated with debridement, rhBMP-2, local administration of a high dose of antibiotic, and systemic administration of a second antibiotic, would form significantly more and stronger new bone than debrided defects treated with rhBMP-2 and local antibiotic alone.

Body

A 6 mm segmental defect was surgically created and stabilized with a polyacetyl plate and 6 Kirschner wires in the left femur in each animal.[1-4] All defects were contaminated with 10^4 colony-forming units (CFUs) of *Staphylococcus aureus*.

Table 1. Experimental design

Treatment			Number of Animals Studied				
rhBMP-2 Dose	Local Antibiotic (Cefazolin)	Systemic Antibiotic (Ceftriaxone)	Total	Time From Debridement			
				2 wk*§	4 wk*§	8 wk*§	12 wk*§
200 μg	yes	yes	72	12†#	20†‡#	20†‡#	20†‡#
		no	72	12†#	20†‡#	20†‡#	20†‡#
20 μg	yes	yes	56	8†	16†‡	16†‡	16†‡
		no	56	8†	16†‡	16†‡	16†‡
0 μg	yes	no	32	8	8	8	8
200 μg	no	no	32	8†	8†	8†	8†
0 μg	no	no	32	8	8	8	8

* radiographic grading of bony lysis: n = 8 animals for each of the 6 treatments at the indicated time-points
† micro-computed tomography: n = 8 animals at the indicated rhBMP-2 treatments/time-points
§ descriptive undecalcified histology: n = 4 animals randomly chosen from among the 8 animals used for the above imaging assessments for each of the 7 treatment at the indicated time-points
‡ torsional failure testing: n = 8 additional animals at the indicated rhBMP-2 treatments/time-points
# quantitative bacteriology: n = 4 additional animals at the indicated rhBMP-2 treatments/time-points

The animals were allowed to recover for 2 weeks during which time the initial contamination progressed to a chronic infection. All defects were then thoroughly debrided under sterile conditions, and treated with a composite type 1 bovine collagen sponge/ceramic-collagen matrix carrier containing

0, 20 or 200 μg of rhBMP-2 in sterile water (used to wet the sponge), with and without a 100 mg dose of Cefazolin dissolved in sterile water (used to wet the matrix). The sponge was wrapped around the matrix and packed into the defect. In half of the animals receiving rhBMP-2 and local Cefazolin, a systemic antibiotic (Ceftriaxone) was also administered for 28 days (intramuscular injection, 50 mg/kg once per day). The rats were then allowed to survive then euthanized at either 2, 4, 8 or 12 week time points (**Table 1**).

The seven treatment groups at 4 different time points were assessed thru the following outcome and endpoint measurements: (i) volume (mm^3) of new bone formation within and outside the defect measured by Micro-Computed Tomography, (ii) Torsional failure testing harvesting the intact contralateral femur as controls, (iii) high resolution radiography to assess pin tract bony lysis, (iv) Qualitative and quantitative bacteriology by means of culture swabs and serial dilution method of counting bacteria (log_{10}) respectively, and finally (v) Stevenel's blue and Van Gieson picrofuchsin stained undecalcified histology readings.

The complete micro-CT for the 12-week group and histology data for the 8 and 12 week groups of rats were received from subcontracters at University of Minnesota and Mayo Clinic on 1/30/09 and will be presented in the forthcoming manuscript.

Micro-Computed Tomography

Micro-CT results (2, 4 and 8 week) revealed that BMP maintained its osteoinductive capability despite the presence of chronic infection and colonized hardware (**Figures 1 and 2**). No substantial callus formed in the chronically infected defects without a sufficiently high dose of BMP.

Figure 1. 3-D rendering (left) and sectional view (right) of representative micro-CT scan of defect treated with 200 μg of rhBMP-2 with both local and systemic antibiotic at 4 weeks; note the segment of ceramic collagen matrix remaining in the defect

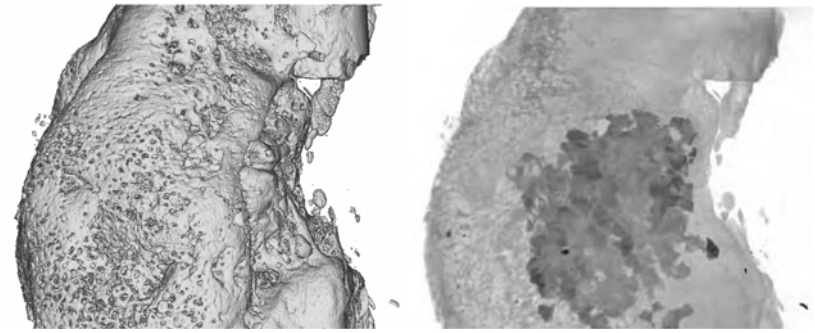
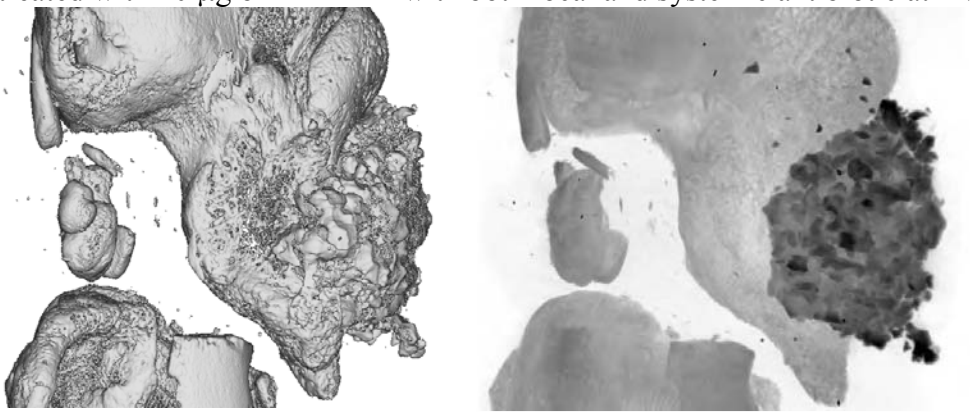


Figure 2. 3-D rendering (left) and sectional view (right) of representative micro-CT scan of defect treated with 20 μg of rhBMP-2 with both local and systemic antibiotic at 4 weeks



The volume, surface area, surface area-to-volume ratio and average grayscale of the total amount of newly mineralized callus formed within the defect and bridging the outside of the defect as well as the volume of the ceramic collagen matrix carrier remaining in the defect and percentage of new callus formed within the defect, are presented in **Table 2**.

Table 2. Micro-CT data summary*

CT Parameters	Treatments				
	20+L-S	20+L+S	200-L-S	200+L-S	200+L+S
Total Volume (mm^3)					
2 Week	21 (16)‡	25 (13)‡	17 (17)‡	34 (22)	46 (20)§
4 Week	43 (54)	19 (19)	23 (16)	43 (41)	70 (28)
8 Week	43 (32)	57 (33)	48 (50)	73 (34)	92 (29)
12 Week†	89 (46)	80 (45)	78(60)	87(43)	96(34)
Matrix Volume (mm^3)					
2 Week	18 (7)	13 (5)	13 (11)	13 (8)	21 (6)
4 Week	12 (4)	18 (10)	10 (10)	12 (6)	20 (5)
8 Week	9 (5)ξ	17 (12)	9 (6)	12 (5)	18 (6)
12 Week†	-	-	-	-	-
Total Surface Area (mm^2)					
2 Week	598 (556)‡	584 (203)‡	422 (398)‡	1046 (723)	1324 (602)
4 Week	936 (1007)	478 (573)	418 (299)‡	1183 (1089)	1909 (879)
8 Week	619 (346)‡	945 (560)‡	764 (757)‡	1313 (925)	1904 (763)
12 week†	-	-	-	-	-
Surface Area/Volume Ratio					
2 Week	26 (7)	27 (8)	31 (14)	29 (7)	29 (5)
4 Week	26 (4)	22 (5)	19 (4)	29 (6)	27 (4)
8 Week	17 (5)	16 (3)	17 (3)	17 (4)	20 (3)
12 week†	-	-	-	-	-
Average Grayscale					
2 Week	1783 (95)§	1827 (124)§	1790 (90)§**	1793 (191)§	1710 (78)§
4 Week	1848 (91)§	2051 (166)¶	2006 (148)¶	1895 (156)§	1735 (64)§
8 Week	2151 (157)	2175 (299)	2090 (239)	2116 (214)	1990 (141)
12 week†	-	-	-	-	-
% New Callus Within Defect					
2 Week	52 (24)	54 (12)	35 (18)	25 (14)	28 (14.)
4 Week	63 (31)	70 (26)	61 (17)	45 (18)	37 (18)
8 Week	59 (18)	54 (18)	48 (22)	31 (11)	32 (11)
12 Week†	-	-	-	-	-

* Data shown as mean (standard deviation); 20 or 200 μg rhBMP-2,
L = Local antibiotic, S = Systemic antibiotic

† These data are in the process of statistical analysis which will be presented in the manuscript for future publication

‡ Significantly less than treatment with 200+L+S at same time point ($p<0.045$, one-way analysis of variance)

§ Significantly less than same treatment at 8 weeks ($p<0.045$, one-way analysis of variance)

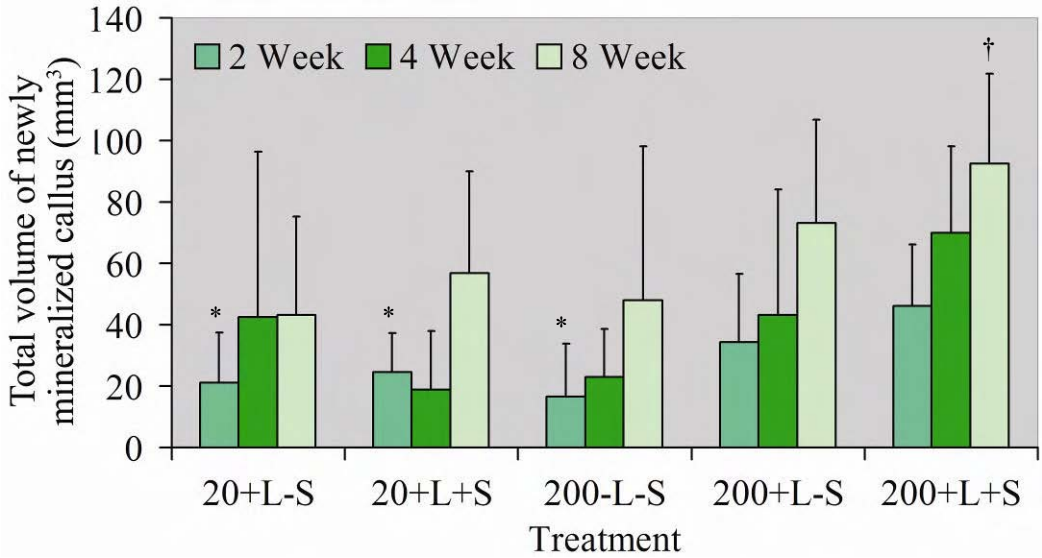
ξ Significantly less than same treatment at 2 weeks ($p=0.026$, one-way analysis of variance)

¶ Significantly greater than 200+L+S at the same time point ($p<0.009$, one-way analysis of variance)

** Significantly less than same treatment at 4 weeks ($p=0.032$, one-way analysis of variance)

The greatest amount of new bone formation occurred with 200 μg of rhBMP-2 augmented with both local and systemic antibiotic (**Figure 3, Table 2**). The total volume and total surface area with the 200 μg dose of rhBMP-2 with local and systemic antibiotic were greater than with 200 μg of rhBMP-2 with systemic antibiotic alone, and 200 μg of rhBMP-2 with no antibiotic. The mean total volume and total surface area were greater with 200 μg of rhBMP-2 than with 20 μg (**Figure 3, Table 2**). There was a clear dose response relationship between rhBMP-2 and bone formation, and application of local and systemic antibiotic affected this relationship in a positive way. The mean total volume, total surface area and average grayscale of newly formed callus increased with time from debridement for all treatment groups except for the total surface area for the 20 μg rhBMP-2 group with local antibiotic only (**Figure 3, Table 2**). The ceramic collagen matrix carrier for the delivery of local antibiotic was not resorbed by 8 weeks after debridement (**Figures 1 and 2, Table 2**). Approximately half of the newly mineralized callus formed within the volume of the original defect (**Table 2**).

Figure 3. Total volume of newly mineralized callus:
20 or 200 μg rhBMP-2, L = Local antibiotic, S = Systemic antibiotic



*Significantly less than 200+L+S ($p<0.045$) †8 weeks significantly greater than 2 weeks ($p=0.003$)

Torsional Failure Testing

Treated and intact femurs were loaded to torsional failure in a materials test machine (MTS Systems, Inc., Eden Prairie MN) (**Figure 4, Table 3**). Torque versus angular displacement data were recorded and used to compute torque to failure, energy absorbed to failure, and torsional stiffness of the newly mineralized callus.

Figure 4. Illustration of failure testing of a segmental defect with callus formation.
A section of the fixation plate was removed so as to not influence failure testing.

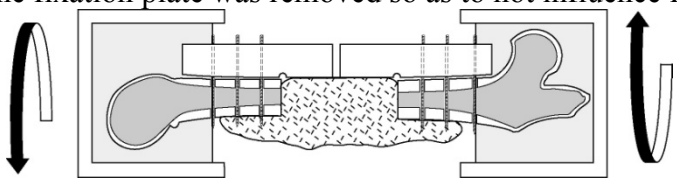


Table 3. Torsional failure data summary*

Mechanical Parameters	Treatments**			
	20+L-S	**20+L+S**	**200+L-S**	**200+L+S**
Maximum Torque (Nm)				
4 Week				
Treatment	-	-	0.149 (0.029)	0.162 (0.087)
Intact	-	-	-	-
8 Week				
Treatment	0.094 (0.135)†‡	0.045	0.243 (0.181)†	0.376 (0.157)
Intact	0.371 (0.095)	0.459 (0.102)	0.547 (0.170)	0.478 (0.099)
12 Week				
Treatment	`0.079 (0.091)†	0.194 (0.214)†	0.199 (0.224)†	0.340 (0.257)
Intact	0.384 (0.101)	0.422 (0.062)	0.413 (0.071)	0.434 (0.092)
Energy (Nm-deg)				
4 Week				
Treatment	-	-	0.825 (0.086)	1.543 (1.136)
Intact	-	-	-	-
8 Week				
Treatment	0.496 (0.654)‡	0.206 (0.261)†‡	1.031 (0.862)†	1.716 (0.756)†
Intact	1.453 (0.703)‡§	2.531 (1.917)	4.046 (1.337)	3.351 (1.348)
12 Week				
Treatment	1.576 (1.942)	1.715 (2.517)	1.229 (1.408)†	1.620 (1.224)
Intact	3.035 (1.101)	3.295 (0.798)	3.591 (2.791)	3.246 (1.496)
Stiffness (Nm/deg)				
4 Week				
Treatment	-	-	0.0196	0.0254
Intact	-	-	-	-
8 Week				
Treatment	0.0212	0.0118	0.0395 (0.0336)	0.0692
Intact	0.0652 (0.0111)ξ	0.0699 (0.0224)ξ	0.0560 (0.0158)	0.0435
12 Week				
Treatment	0.0061†	0.0233†	0.0285 (0.0349) †	0.0559
Intact	0.0374 (0.0117)	0.0542 (0.0240)	0.0459 (0.0106)	0.0435

* Data shown as mean (standard deviation)

** 20 or 200 μg rhBMP-2, L = Local antibiotic, S = Systemic antibiotic

- Indicates that there was not enough healing to resist torque

† Significantly less than respective intact contralateral femur ($p<0.047$, paired Student's t-test)

‡ Significantly less than 200+L+S ($p<0.030$, one-way analysis of variance)

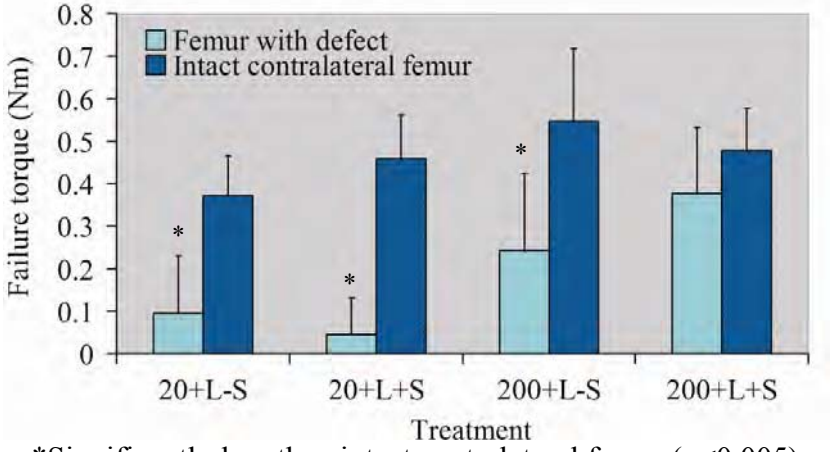
§ Significantly less than 200+L-S ($p<0.032$, one-way analysis of variance)

ξ Significantly greater than 200+L+S ($p<0.041$, one-way analysis of variance)

Many of the specimens that were to be mechanically tested exhibited new bone formation in and around the defect as shown by the micro-CT data, but this new bone did not sufficiently unite the ends of the defect so as to resist the applied torque. This was especially evident in the 4-week animals. The mean failure strength of the 8 and 12-week treatment group with 200 μg of rhBMP-2 in combination with local and systemic antibiotic was not significantly different than the mean strength of the respective intact contralateral femurs (**Figure 5 and 5-1**, $p>0.05$, paired Student's t-test). On the other hand, the mean failure strengths of defects in the other three treatment groups (20 μg of rhBMP-2 with local antibiotic with/without systemic antibiotic, and 200 μg rhBMP-2 with local antibiotic only) were significantly less than their intact counterparts at 8 and 12 weeks ($p<0.001$, paired Student's t-test).

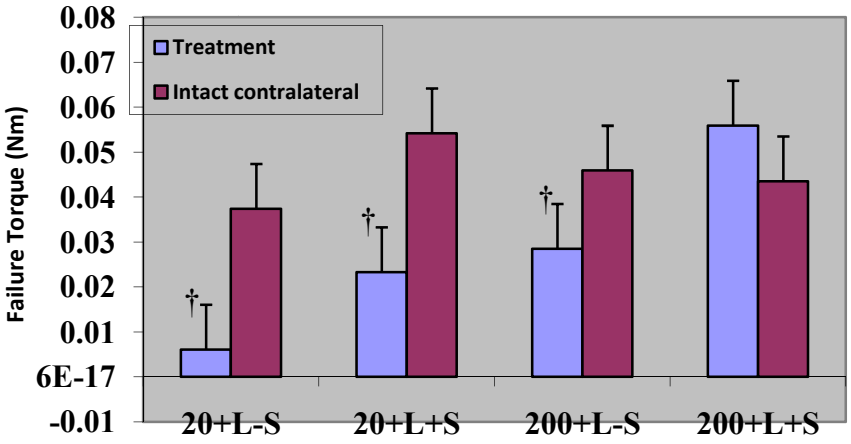
The group with 200 μg rhBMP-2 in combination with local and systemic antibiotic exhibited the highest mean mechanical strength of any of the treatments at 8 and 12 weeks (**Table 3**). The mean torsional failure strength in this treatment group was significantly greater than the two 20 μg rhBMP-2 groups ($p<0.003$, one-way analysis of variance).

Figure 5. Failure torque (mean, standard deviation) at 8 weeks as a function of rhBMP-2 dose + antibiotic therapy: 20 or 200 μg rhBMP-2, L = Local antibiotic, S = Systemic antibiotic



*Significantly less than intact contralateral femur ($p<0.005$)

Figure 5-1. Failure torque (mean, standard deviation) at 12 weeks as a function of rhBMP-2 dose + antibiotic therapy: 20 or 200 μg rhBMP-2, L = Local antibiotic, S = Systemic antibiotic



†Significantly less than intact contralateral femur ($p<0.005$)

Bony Lysis

Bone damage in infected segmental defects initially presents on high resolution radiographs as cortical osteolysis adjacent to the Kirschner wires as they cross the cortical bone (**Figures 6 and 7**).[3,4] A simple count of the number of sites of lysis (6 Kirschner wires crossing cortical bone twice, or 12 possible sites) has been shown to correlate with the torsional stiffness of the defect fixation.[1]

Figure 6. Illustration of osteolysis in segmental defect model with chronic infection

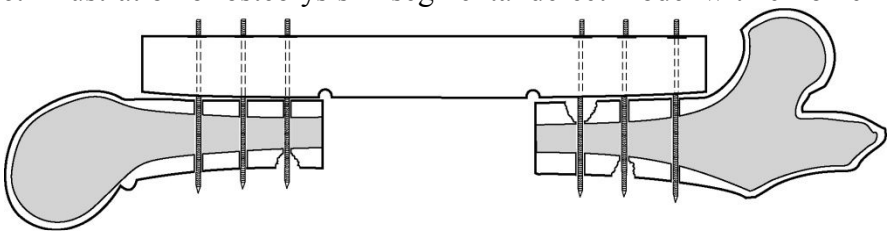
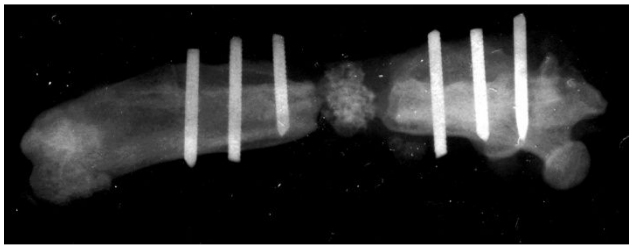


Figure 7. Representative radiograph showing osteolysis in the segmental defect model



High resolution faxitron radiographs were scanned and digital formatted then analyzed. The numbers of sites of osteolysis are summarized in **Table 4** below. At least some level of osteolysis occurred over the study period regardless of treatment group. This suggests that the infection was truly chronic and could likely not be completely eliminated without removal of the fixation implant. There was a large amount of variation in the occurrence of bony lysis among the specimens which reflects the inherent variability of infection whether in animal models or clinically. The median number of sites of lysis was lowest when the infected defects were treated with 200 μg of BMP-2, together with both local and systemic antibiotic. Addition of systemic antibiotic to the local administration of antibiotic reduced the occurrence of lysis with both 20 and 200 μg of rhBMP-2, although this was not statistically significant. The median number of sites of lysis was lower with 200 μg of rhBMP-2 than with 20 μg, for a given antibiotic treatment (local ± systemic), which suggests the possibility that rhBMP-2 may somehow be involved with modulating the infective process.

Table 4. Median number of sites of bony lysis from high resolution radiographs

Treatment			Number of Sites of Lysis*			
rhBMP-2 Dose	Local Antibiotic (Cefazolin)	Systemic Antibiotic (Ceftriaxone)	Time From Debridement			
			2 wk	4 wk	8 wk	12 wk
200 μg	yes	yes	2 (3)	2 (2.25)	0 (1)	2 (2.5)
		no	4 (5.5)	2.5 (2)	1.5 (2)	4 (3.5)
20 μg	yes	yes	3.5 (3)	6 (5.5)	3 (3)	3 (1.5)
		no	3 (4.5)	9.5 (6.5)†	6 (1.75)†	5 (5.5)
0 μg	yes	no	8 (4.5)	6 (6.5)	6 (1.5)†‡	6.5 (3.5)
200 μg	no	no	5 (5)	5 (5)	5 (3.5)†‡	5.5 (5)
0 μg	no	no	5 (2.25)	6 (5)	5 (0)†	8 (4)†§

* Data shown as median (interquartile range); data were not normally distributed so Kruskal-Wallis one way analysis of variance on ranks was used, with multiple pairwise comparisons made with Dunn's Method

† Significantly greater than 200μg + local and systemic antibiotic ($p < 0.05$)

‡ Significantly greater than 200μg + local antibiotic ($p < 0.05$)

§ Significantly greater than 20μg + local and systemic antibiotic ($p < 0.05$)

Qualitative Bacteriology

The defects in all animals became infected by 2 weeks after contamination with 10^4 CFUs of *Staphylococcus aureus*, except for three 8-week animals (**Table 5**). These animals were replaced by three others whose defects did become infected by 2 weeks after contamination. The majority of the cultures from infected defects revealed moderate to many CFUs of *S. aureus*, and occasionally, only a few CFUs were evident, although the defect showed clear clinical signs of infection; predominately pus and inflammation.

Table 5. Qualitative bacteriology at time of *debridement*

Culture Results	Treatment						
	0-S-L	200-S-L	0+L-S	20+L-S	20+L+S	200+L-S	200+L+S
2 Weeks							
No growth							
Rare							
Few						1	
Moderate	3	2	3	3	2	4	6
Many	5	5	1		3	4	4
Contaminated*			3	5	3		
4 Weeks							
No growth							
Rare							
Few		2					
Moderate	2	4	1	1	2	2	6
Many	5	1	6	12	11	17	13
Contaminated*		1	1	3	3	1	1
8 Weeks							

No growth							3
Rare							
Few	1			2		1	4
Moderate	5	4	6	6	7	7	4
Many	2	4	2	7	9	12	10
Contaminated*							
12 Weeks †							
No growth	-	-	-	-	-	-	-
Rare	-	-	-	-	-	-	-
Few	-	-	-	-	-	-	-
Moderate	-	-	-	-	-	-	-
Many	-	-	-	-	-	-	-
Contaminated*	-	-	-	-	-	-	-

20 or 200 μg rhBMP-2, L = Local antibiotic, S = Systemic antibiotic

* Contamination with another type of bacteria in addition to *Staphylococcus aureus*

† These data are unavailable. Rats during debridement were clinically and grossly infected.

Qualitative cultures at the time of euthanasia revealed continued bacterial growth in 71% of the defects in the 200 μg rhBMP-2 + local antibiotic group (**Table 6**). Addition of systemic antibiotic therapy to this treatment group decreased the number of defects with bacterial growth at euthanization by half (39%). This reduction in the occurrence of chronic infection from administration of both local and systemic antibiotic was not evident in the 20 μg rhBMP-2 groups. The remaining 3 treatment groups exhibited bacterial growth in 100% of the defects. Thus, qualitative cultures revealed that the chronic infection remained in evidence throughout the study period in the majority of defects, and that administration of both local and systemic antibiotic therapy with 200 μg rhBMP-2 decreased the number of defects with infection. These data also reflect the inherent variability with infection and suggest the possibility that the presence of rhBMP-2 itself may have somehow played a role in modulating the infection as evidenced by the different effects in the cultures with the 20 and 200 μg rhBMP-2 groups for a given antibiotic treatment (local ± systemic).

Table 6. Qualitative bacteriology at time of *euthanasia*

Culture Results	Treatment*						
	0-S-L	200-S-L	0+L-S	20+L-S	20+L+S	200+L-S	200+L+S
2 Weeks							
No growth					1		8
Rare			1	1	1		2
Few	1		3	3	3	5	
Moderate	5	3		1		2	
Many	2	2				1	
Contaminated†		2	3	2	3	1	
4 Weeks							
No growth				3	1	3	7
Rare	1		3	4	3	7	5
Few	1	1	2	2	2	6	5
Moderate	3	2	2	3	1	2	2
Many	2	2		2		1	
Contaminated†		3	1	1	8	1	1
8 Weeks							

No growth				7	5	11	16
Rare	1			3	4	2	3
Few	2	5	3		5	3	2
Moderate	3		5	2	2	3	
Many	2	1		3			
Contaminated†		2				1	
12 Weeks							
No growth		5	2	12	8	16	16
Rare					2	1	
Few	2	3	1	2		1	2
Moderate	6		5		1		
Many							
Contaminated†					2		

* 20 or 200 μg rhBMP-2, L = Local antibiotic, S = Systemic antibiotic

† Contamination with another type of bacteria in addition to *Staphylococcus aureus*

Quantitative Bacteriology

S. aureus was recovered from the femurs with an infected defect even though they were treated with local and systemic antibiotics, as shown in **Table 7**. Treatment of infected defects with 200 μg BMP-2 with both local and systemic antibiotics led to numbers of recovered bacteria that were 4 logs less (at the same level as the contaminating inoculum) than with treatment with 200 μg rhBMP-2 with local antibiotic alone. As was also observed with the qualitative bacteriology, both systemic and local antibiotics were required to substantially impact the chronic infection. Statistical analysis was not performed on these data because there were only 4 samples per group. The intent of these measurements was to simply monitor the progression and magnitude of the infection in a few animals.

Table 7. Bacterial census measurements*

Treatment†	Time from debridement (wks)		
	4	8	12
200 μg BMP-2 + L	7.88 (8.12)	8.24 (8.18)‡	7.87 (7.63)‡
200 μg BMP-2 + L + S	6.94 (7.22)	3.94 (3.43)	8.31 (8.24)‡

* Data presented as $\log_{10}$ of mean (standard deviation) of number of recovered CFUs of bacteria (n = 4 per treatment and time point)

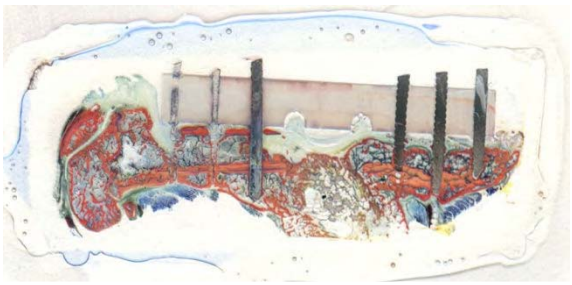
† L = local antibiotic and S = systemic antibiotic

‡ Significantly greater than 200 μg BMP-2 + L + S at 8 weeks ($p<0.05$); One way analysis of variance on log transformed data, with multiple comparisons made using the Holm-Sidak method

Undecalcified Histology

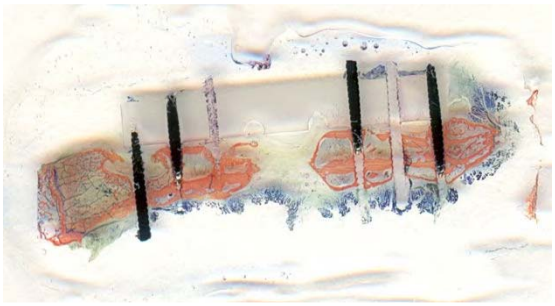
Figure 8 shows representative slides of the 2 and 4 week group. The Van Gieson picrofuchsin stained collagen fibers green, bone orange and osteoid yellow-green. Stevenel's blue stained cells and extracellular structures in blue tones. The histological sections from the 8 & 12-week animals were received January 2009 from University of Minnesota subcontractor, and are currently being analyzed. Results will be available upon completion of the manuscript for publication.

Figure 8. Representative undecalcified sections at 4 weeks

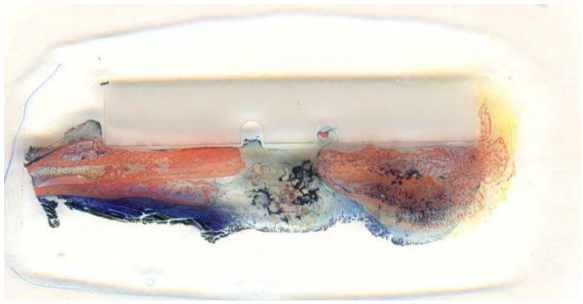


200 μg BMP-2 + local + systemic abx

200 μg BMP-2 + local abx



20 μg BMP-2 + local + systemic abx



20 μg BMP-2 + local abx

The histology findings are summarized in **Table 8**. The greatest amount of new bone formation at 4 weeks occurred with 200 μg of rhBMP-2 augmented with both local and systemic antibiotic.

Table 8. Summary of histological findings:
0, 20 or 200 μg rhBMP-2, L = Local antibiotic, S = Systemic antibiotic

Treatment Group	Description of Histology
2 Week Group	
0-L-S	No new bone formation
0+L-S	No new bone formation
20+L-S	There is scant new bone formation admixed within the matrix carrier surrounded by collagen fibers
200-L-S	No new bone formation
200+L-S	There is scant new bone formation admixed within the matrix carrier surrounded by collagen fibers
20+L+S	There is scant new bone formation admixed within the matrix carrier surrounded by collagen fibers
200+L+S	There is minimal to moderate new bone formation in and around the matrix carrier

4 Week Group	
0-L-S	No new bone formation
0+L-S	No new bone formation
20+L-S	There is minimal new bone formation admixed within the matrix carrier surrounded by collagen fibers
200-L-S	No new bone formation
200+L-S	Minimal new bone formation can be seen within and around the matrix carrier surrounded by collagen fibers.
20+L+S	There is scant new bone formation within the matrix carrier surrounded by collagen fibers
200+L+S	There is moderate new bone formation in and around the matrix carrier with more organized creeping substitution

Key Research Accomplishments

● Using an animal model with an internally-stabilized segmental defect in the rat femur with a chronic infection from *Staphylococcus aureus*, we learned that …

► Debridement and use of commercially-available off-the-shelf biologics and antibiotics led to formation of newly mineralized callus in spite of retention of the colonized hardware

► Recombinant human bone morphogenetic protein-2 with its absorbable collagen sponge carrier maintained its osteoinductive capability despite the presence of chronic infection and colonized hardware.

► There was a clear dose response relationship between rhBMP-2 and bone formation, and application of local and systemic antibiotic affected this relationship in a positive way. More newly mineralized callus formed with the higher dose of rhBMP-2 (200 μg) than the lower dose (20 μg). The greatest amount of new bone formation occurred with 200 μg of rhBMP-2 augmented with both local and systemic antibiotic.

► The bone-forming capability of rhBMP-2 in the presence of chronic infection was enhanced by antibiotic therapy. More newly mineralized callus formed with rhBMP-2 and antibiotic introduced local to the defect, compared to defects with rhBMP-2 without local antibiotic. The greatest amount of newly mineralized callus was induced with rhBMP-2 augmented with both local and systemic antibiotic.

► The strength of the newly mineralized callus formed within and bridging the defect with the high dose of rhBMP-2 in combination with local and systemic antibiotic approached the strength of the intact contralateral femurs. The mechanical strength of all other treatment groups was significantly less than the respective intact contralateral femurs.

Reportable Outcomes

● Animal model of an internally-stabilized segmental defect in the rat femur with a chronic infection from *Staphylococcus aureus* in which debridement and use of commercially-available off-the-shelf biologics (bone morphogenetic protein-2, absorbable collagen sponge (ACS), ceramic collagen matrix

(MasterGraft Matrix)) and antibiotics lead to formation of newly mineralized callus in spite of retention of the colonized hardware

- A poster presentation was done at the Society Of Military Orthopaedic Surgeon (SOMOS) December 2008 meeting and an abstract has been accepted as a short-talk poster in the upcoming Orthopaedic Research Society meeting in February 2009. A manuscript is forthcoming and will be submitted to the *Journal of Orthopaedic Trauma* for publication.

Conclusions

The treatment of an infection at the site of a fracture often necessitates removal of internal fixation. However, internal fixation is needed for fracture stability. This study presents an intervention that may accelerate fracture-healing in the presence of chronic infection and colonized hardware, thereby permitting earlier removal of the hardware and more timely and effective treatment of the infection.

The results of this study demonstrated that rhBMP-2 maintained its osteoinductive capability despite the presence of chronic infection and colonized hardware, and this property was enhanced by local and systemic antibiotic. No significant new bone was formed unless rhBMP-2 was introduced. A composite type 1 bovine collagen sponge/ceramic-collagen matrix carrier containing 200 μg of rhBMP-2 in sterile water (used to wet the sponge) and 100 mg of Cefazolin dissolved in sterile water (used to wet the matrix) was applied to surgically debrided defects, together with 4 weeks of systemic administration of the antibiotic Ceftriaxone. This treatment induced a substantial amount of newly mineralized callus that connected the ends of the defect at 8 weeks after debridement such that there was no significant difference between the torsional failure strength of these treated defects and the intact contralateral femurs.

There was also a clear dose response relationship between rhBMP-2 and bone formation, and application of local and systemic antibiotic affected this relationship in a positive way. More new bone was formed when both systemic and local antibiotic were administered, compared to when local antibiotic was introduced alone. The lower 20 μg dose of rhBMP-2 also led to the formation of newly mineralized callus, particularly when local and systemic antibiotics were administered, but the amount of new bone was less than with the higher 200 μg dose of rhBMP-2. Although newly formed callus was induced in the 20 μg rhBMP-2 defects, this new bone and surrounding inflammatory fibrous tissue did not sufficiently unite the ends of the defects to resist the torque applied with mechanical testing. It is clear from this experiment that the combination of both local and systemic antibiotic and high dose rhBMP-2 had the greatest effect achieving bridging bone across the segmental defect - the desired clinical state. There is likely an optimal dose of rhBMP-2 that attains some threshold for adequate stimulation of bone formation in the presence of chronic infection, but this remains to be determined. The dose is also likely dependent on phylogenetic considerations. This has been seen in non-infected studies in various order animals. The molecular and genetic mechanisms involved in the interaction of rhBMP-2, antibiotic and infection are unknown at this point, and should be the subject of future work.

The logical next steps for this work are to assess the efficacy of rhBMP-2 with local and systemic antibiotic in a polymicrobial chronic infection model in the rat, more closely simulating combat injuries, and then develop a similar chronic infection model in a large animal (e.g. goat), which would more closely approximate the human condition.

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Personnel Receiving Pay From the Grant

David W. Polly Jr, MD: Principal Investigator
Charles Ledonio, MD: Animal surgeon
Joan E. Bechtold, PhD: Co-Investigator
Dean T. Tsukayama, MD: Co-Investigator
William D. Lew, MS: Investigator
Barbara W. Wicklund, BS: Bacteriologist
Brooke Sommer: Animal care technician

Appendices

SOMOS 2008: Poster
Orthopaedic Research Society 2009: Abstract accepted as short-talk poster presentation

Supporting Data

All supporting data is embedded in the body of the report

NEW BONE FORMATION IN CHRONICALLY-INFECTED SEGMENTAL DEFECT IN RAT FEMUR TREATED WITH BMP-2 AND LOCAL AND SYSTEMIC ANTIBIOTIC

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Introduction

Most combat casualties are a result of high-energy blast or high-velocity projectile mechanisms and commonly result in the following:

- Significant bone defects
- Massive tissue damage
- Substantial bacterial infection

Specific Aim

To determined whether recombinant human bone morphogenetic protein-2 (rhBMP-2) and antibiotic delivered locally from a composite carrier, in combination with a second antibiotic administered systemically, could lead to new bone formation in an internally-stabilized rat femoral segmental defect with a chronic infection from Staphylococcus aureus.

Method

A 6 mm segmental defect was surgically created and stabilized with a polyacetyl plate and 6 threaded Kirschner wires in the left femur of 256 Sprague-Dawley rats. All defects were contaminated with 10^4 colony-forming units of Staphylococcus aureus. The animals were allowed to recover for 2 weeks while the contamination progressed to a chronic infection.

All defects were then thoroughly debrided under sterile conditions, and treated with a composite type 1 bovine collagen sponge/ceramic-collagen matrix carrier containing 0, 20 or 200μg of rhBMP-2 in sterile water with and without 100 mg of Cefazolin dissolved in sterile water. The sponge was wrapped around the matrix and packed into the defect. In half of the animals receiving rhBMP-2 and local Cefazolin, a systemic antibiotic (Ceftriaxone 50mg/kg) was also administered for 28 days. Outcome measures are listed in Table 1.

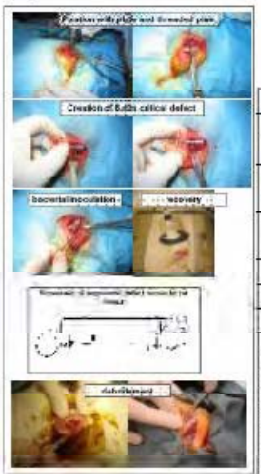


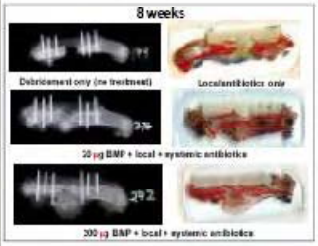
Table 1. Experimental Design

Treatment			Number of Animals Studied			
				Time From Debridement		
rhBMP-2 Dose	Local Antibiotic	Systemic Antibiotic	Total	2 wks*§	4 wks*§	8 wks*§
200μg	yes	yes	72	12†#	20†‡#	20†‡#
		no	72	12†#	20†‡#	20†‡#
20μg	yes	yes	56	8†	16†‡	16†‡
		no	56	8†	16†‡	16†‡
0μg	yes	yes	32	8	8	8
200μg	no	no	32	8	8	8
0μg	no	no	32	8	8	8

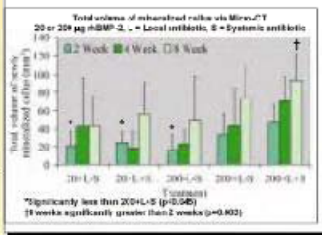
* radiographic grading of bony bytic: n = 8 animals for each of the 8 treatments at the indicated time-points
† micro-computed tomography: n = 8 animals at the indicated rhBMP-2 treatments/time points
‡ descriptive undecalcified histology: n = 4 animals randomly chosen from among the 8 animals used for the above imaging assessments for each of the 7 treatment at the indicated time-points
§ torsional failure testing: n = 8 additional animals at the indicated rhBMP-2 treatments/time points
# quantitative bioterfology: n = 4 additional animals at the indicated rhBMP-2 treatments/time points

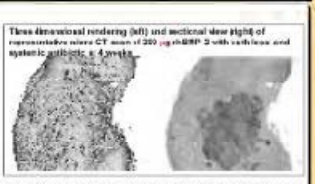
Results

The greatest amount of new bone formation occurred with 200 μg of rhBMP-2 augmented with both local and systemic antibiotic.

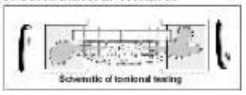


The total volume of mineralized callus with 200 μg of rhBMP-2 with local and systemic antibiotic was greater than with 200 μg of rhBMP-2 with local antibiotic alone, and 200 μg of rhBMP-2 with no antibiotic. The total volume of new bone was greater with 200 μg of rhBMP-2 than with 20 μg.

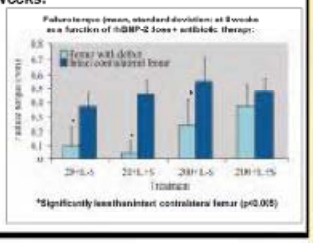




The mean failure strength of the 8-week treatment group with 200 μg of rhBMP-2 in combination with local and systemic antibiotic was not significantly different than the mean strength of the respective intact contralateral femurs.



On the other hand, the mean failure strengths of defects in the other three treatment groups (20 μg rhBMP-2 with local antibiotic with/without systemic antibiotic, and 200 μg rhBMP-2 with local antibiotic only) were significantly less than their intact counterparts at 8 weeks.



Conclusions

- High dose BMP with local and systemic antibiotic therapy induced a substantial amount of newly mineralized callus connecting the ends of a chronically infected segmental bone defect at 8 weeks after debridement such that there was no significant difference between the torsional failure strength of these treated defects and the intact contralateral femurs.
- Debridement and use of commercially-available off-the shelf biologics and antibiotics led to new bone formation in spite of chronic infection with retention of colonized hardware.
- RhBMP-2 maintained its osteoinductive capability in the presence of chronic infection, and this property was enhanced by local and systemic antibiotic.
- No significant new bone was formed unless rhBMP-2 was introduced.
- This study presents an intervention that may accelerate fracture-healing in the presence of chronic infection and colonized hardware.
- Confirmatory human clinical studies are needed and regulatory status must be considered.

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New Bone Formation in Chronically-Infected Segmental Defect in Rat Femur Treated with BMP-2 and Local and Systemic Antibiotic

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Introduction: The majority of combat casualties in Iraq and Afghanistan are wounds to the extremities resulting from high-energy blasts or projectiles, and commonly present with a significant segmental bone defect, soft tissue disruption and bacterial contamination. The goal of this study was to determine whether recombinant human bone morphogenetic protein-2 (rhBMP-2) and antibiotic delivered locally from a composite carrier, in combination with a second antibiotic administered systemically, could lead to new bone formation in an internally stabilized rat femoral segmental defect with a chronic infection from *Staphylococcus aureus*.

Methods: This study was approved by the Institutional Animal Care and Use Committee. A 6 mm segmental defect was surgically created and stabilized with a polyacetyl plate and 6 threaded K-wires in one femur of 256 Sprague-Dawley rats. All defects were contaminated with 10^4 colony-forming units of *S. aureus*. The animals were allowed to recover for 2 weeks while the contamination progressed to a chronic infection. All defects were then thoroughly debrided under sterile conditions, and treated with a composite type 1 bovine collagen sponge/ceramic collagen matrix carrier containing 0, 20 or 200 μg of rhBMP-2 in sterile water (used to wet the sponge), with and without 100 mg of Cefazolin dissolved in sterile water (used to wet the matrix). The sponge was wrapped around the matrix and packed into the defect. In half of the animals receiving rhBMP-2 and local Cefazolin, a systemic antibiotic (Ceftriaxone) was also administered for 28 days. The animals were euthanized at 2, 4 and 8 weeks after debridement. Newly mineralized callus was assessed with micro-computed tomography, torsional failure testing and undecalcified histology. The progression of the chronic infection was assessed with quantitative bacteriology and bony lysis from high resolution radiographs.

Results: The greatest amount of newly mineralized callus occurred with 200 μg rhBMP-2 augmented with both local and systemic antibiotic (Figs. 1-3). The total volume of callus with 200 μg rhBMP-2 with local and systemic antibiotic was greater than with 200 μg rhBMP-2 with local antibiotic alone and with no antibiotic. The total volume of new bone was greater with 200 μg rhBMP-2 than with 20 μg. There was a clear dose response relationship between rhBMP-2 and bone formation, and application of local and systemic antibiotic affected this in a positive way. The mean failure strength of the 8-week treatment group with 200 μg rhBMP-2 with local and systemic antibiotic was not significantly different than respective intact contralateral femurs (Fig. 4). On the other hand, the mean failure strengths of defects in the other three rhBMP-2 treatment groups in Fig. 4 were significantly less than their intact counterparts at 8 weeks. At least some level of osteolysis occurred over the study period regardless of treatment. The median number of sites of lysis was lowest when infected defects were treated with 200 μg BMP-2 with both local and systemic antibiotic. *S. aureus* was recovered from the femurs with an infected defect even though they were treated with local and systemic antibiotics.

Conclusions: A high dose of rhBMP-2 combined with local and systemic antibiotic induced substantial newly mineralized callus that connected the ends of a chronically infected segmental defect at 8 weeks after debridement such that there was no significant difference between the torsional failure strength of these treated defects and the intact contralateral femurs. RhBMP-2 maintained its osteoinductive capability in the presence of chronic infection and colonized hardware, and this was enhanced by local and systemic antibiotic. This study presents an intervention that may result in earlier bony stabilization and accelerated fracture-healing in the presence of chronic infection and colonized hardware, thereby permitting earlier weight bearing, avoiding hardware failure and more timely and effective treatment of infection.

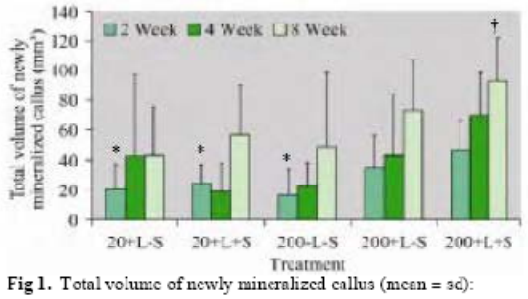

Fig 1. Total volume of newly mineralized callus (mean + sd):
20 or 200 μg rhBMP-2, L = Local antibiotic, S = Systemic antibiotic
*Significantly less than 200+L+S ($p<0.045$)
†8 wks significantly greater than 2 wks ($p=0.003$)

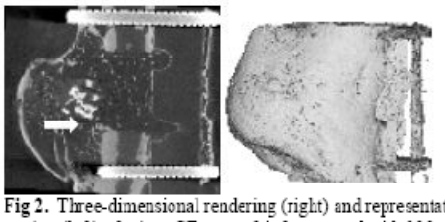

Fig 2. Three-dimensional rendering (right) and representative cross section (left) of micro-CT scan of defect treated with 200 μg rhBMP-2 with both local and systemic antibiotic at 8 weeks; bright area in middle of sectional view is ceramic collagen matrix remaining in defect (arrow)

Fig 3. Histologic images at 8 weeks of defect treated with (left) 20 μg rhBMP-2 with local and systemic antibiotic, and (right) 200μg rhBMP-2 with local and systemic antibiotic. Note the larger amount of callous within and around the defect (arrows).

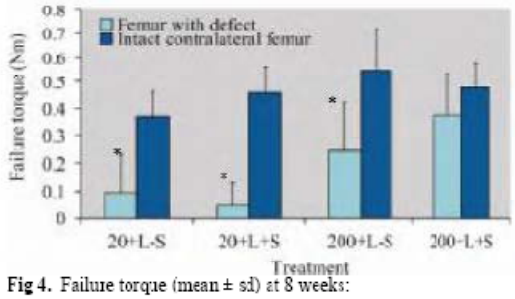

Fig 4. Failure torque (mean ± sd) at 8 weeks:
20 or 200 μg rhBMP-2, L = Local antibiotic, S = Systemic antibiotic
*Significantly less than intact contralateral femur ($p<0.005$)

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